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***IN THE UNITED STATES PATENT AND TRADEMARK OFFICE***

*Group:* 1645  
*Confirmation No.:* 3698  
*Application No.:* 10/084,638  
*Invention:* COMPOSITIONS OF MULTIMERIC  
PROFILIN FOR DIAGNOSIS AND  
TREATMENT OF ALLERGIES  
*Applicant:* Michael Babich.  
*Filed:* February 27, 2002  
*Attorney*  
*Docket:* 21511/92177  
*Examiner:* Nora M. Rooney

Certificate Under 37 CFR 1.8(a)

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(Signature)

Alice O. Martin  
(Printed Name)

December 18, 2006  
Dated

**APPEAL BRIEF**

Mail Stop Appeal Brief-Patents  
Commissioner for Patents  
P.O. Box 1450  
Alexandria, VA 22313-1450

Dear Sir:

Below is an Appeal Brief in support of an appeal taken from the rejection of claims  
17, 22-28 in a Final Office Action mailed January 11, 2006, as amended after Final Rejection  
on June 12, 2006, and entered in the examiner's Advisory Action mailed July 3, 2006.

1. **Real Party in Interest.** All rights in this application have been assigned to  
Immvarx, Inc., a corporation of Illinois whose address is 1009 Johnson Court, Belvidere,

12/21/2006 TTRAN1 Illinois 61008 00000020 10084638

02 FC:2402

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2. **Related Appeals and Interferences.** None.

3. **Status of Claims.**

Claims 17, 22-28 are pending.

Claims 1-16, 18-21, 29-33 were withdrawn.

4. **Status of Amendments.** The amendments filed October 17, 2005 and June 12, 2006 were entered according to the Advisory Action mailed July 3, 2006, after which only claims 17, 22-28 are pending.

5. **Summary of Claimed Subject Matter.**

17. A diagnostic test for allergies, said test comprising:

- (a) obtaining a pharmaceutical composition of multimeric profilin;
- (b) administering the composition to a subject; and
- (c) determining a reaction from which allergenicity is inferred.

6. **Grounds of Rejection to be Reviewed on Appeal.**

A. Whether Claims 17, 22-28 are anticipated by U.S. Patent No. 5,583,046 "as evidenced by Vrtala et al." (Advisory Action)

7. **Argument.**

**I. U.S. Pat. No. 5,583,046 (Valenta et al.,) and Vrtala et al., do not anticipate the pending claims**

A 35 U.S.C. §102 rejection is not supported because all of the steps recited in claim 17 are not taught by the '046 patent and using the Vrtala reference is improper to cure the deficiencies.

On page 2 of the Action, the examiner rejected claims 17 and 22-28 under 35 U.S.C. §102 (b) as being anticipated by U.S. Pat. No. 5,583,046 (Valenta et al), "as evidenced by Vrtala et al." The examiner has not established a legally sufficient basis for a 102 (b) rejection because neither Valenta et al. nor Vrtala et al. either singly or in combination teach all the claimed elements as required to anticipate. In addition, there is no justification for adding Vrtala to what should be a rejection based on a single case. Such a combination is an improper and an incorrect basis for an anticipation rejection. To stretch beyond one reference, the omitted element must be recognized in the art. The examiner has not demonstrated recognition of multimeric profilin as a hyposensitizing

agent. Even if combined, the combination still does not teach all the elements of the pending claims. The examiner's sole reasoning in support of using Vrtala to fill in the admitted deficiencies in Valenta, is as follows:

Vrtala recognized that when Betv2 is placed in solution it naturally polymerizes. The Vrtala et al., reference is only relied upon to characterize an already described process.

Office Action, page 2.

The examiner does not identify what "solution" in Valenta or in the pending application are being compared. There is no proof of an "already described process" that is the same as the claimed process.

**A. Valenta does not teach the claimed elements**

To anticipate, a **single reference must teach all the elements** of the claims. *RCA Corp v. Applied Digital Data Sys., Inc.*, 221 USPQ 385, 388 (Fed. Cir. 1984). An anticipating prior art reference should disclose **each and every limitation** of the claim expressly or inherently. *Akamai Techs. v. Cable & Wireless Internet Servs.*, 344 F.3d 1186, 1192 (Fed. Cir. 2003). To anticipate a claim, a reference must **disclose every element of the challenged claim and enable** one skilled in the art to make the anticipating subject matter. *PPG Industries, Inc. v. Guardian Industries Corp.*, 75 F.3d 1558, 1566, 37 USPQ2d 1618, 1624 (Fed. Cir. 1996). (*emphases added*).

By the examiner's own admission, the '046 (Valenta) patent does not anticipate. The examiner admits that "Claim 17 requires....administering a multimeric profilin" and also admits "The '046 patent.... is silent as to whether Betv2 is multimeric."

As the examiner admits, the '046 patent does not disclose all the claim elements. For example, '046 does not disclose or even suggest the use of a multimeric profilin to hyposensitize a mammal. The '046 patent merely discloses a synthetic version of a 14 kDa birch pollen antigen P14:

The present invention provides **recombinant DNA molecules** which contain a nucleotide sequence that codes for a polypeptide which exhibits the same or similar antigenic properties as a natural allergen, P14,... (Col. 2, lns. 14-17)

The present invention covers the use of **P14 synthetic polypeptide** allergens to hyposensitize or desensitize a

mammal. Such polypeptides can be administered to a human subject either alone or in combination with pharmaceutically acceptable carriers or diluents, in accordance with standard pharmaceutical practice. (Col. 11, Ins. 34-40)

In contrast, the claims of the present application are based, in part, on the increased IgE recognition of profilin multimers, singular fragments based on the sequence that uniquely may arise, or be exposed, upon profilin polymerization that are not available in the monomeric parent molecules. This may reflect one or more novel amino acid sequences that are comprised of part of each of at least two monomers complexed together to form the polymer, or a sequence that is buried within the tertiary monomeric structure that becomes exposed upon multimerization with one or more additional profilins. Such fragments are not dependent upon whether a portion of IgE epitope(s) is present or not. The novel polymers of the present invention takes advantage of native configurations/structural phenomenon that lead to the pan-allergenic potential (not taught in the '046 patent) that, in turn, may be used for diagnostic and therapeutic use to induce a hypoallergenic response.

**B.     The examiner has not countered applicant's assertion that Vrtala teaches away from the claimed invention**

According to the examiner, Claim 17 requires an *in vivo* diagnostic test comprising administering a multimeric profilin molecule. "The '046 patent teaches administering Bet v2, a profilin. The reference is silent as to whether Bet v2 is multimeric." The examiner added Vrtala because of the statement that when rBet v2 is placed in solution, it naturally polymerizes.

There is no teaching in the '046 patent that when Bet v2 is placed in solution, as required to administer to a subject in non-lyophilized form, it polymerizes due to the physical properties of the molecule. The Vrtala reference teaches on page 914 "it could be shown that rBet v2 formed polymers through disulfide bonds" and that "The tendency of recombinant Bet v 2 to form polymers through disulfide bonds under non-reducing conditions was demonstrated by SDS-PAGE, immunoblotting and blot overlays. The examiner's position is that "this reference has been used simply to illustrate an already described process showing inherent properties of the molecule."

Vrtala **teaches against** the present invention. For example, on page 914, left column, Vrtala states the following:

It could be shown that rBet v2 formed polymers through disulfide bonds, and it is hence suggested that the **decreased allergenicity of rBet v 2 might be related to its tendency to polymerize.** (emphasis added)

On page 920, left column, Vrtala further states the following:

[a]nd it is hence possible that the **weaker capacity** of rBet v 2 to induce IgE antibodies might be linked to the ability to form natural **polymers through disulfide bonds.** Although it must be stressed that there is currently no feasible experimental data suggesting that polymerization of antigens might be a mechanism with which to reduce the allergenicity of protein antigens in favor of a TH1 response. (emphasis added)

Therefore, Vrtala teaches away from the present invention which claims profilin multimers result in **increased** allergenicity. The utility of profilin multimers was not recognized in the references cited by the examiner nor are there arguments presented to pinpoint where in solutions of the publication multimeric profilin is formed, and to equate such multimers to those in the present claims.

Vrtala et al. did not find utility for profilin multimers in allergy diagnostics nor therapeutics; they state the opposite.

Their experimental approach does not indicate that profilin multimers would be more allergenic/antigenic and yield possible unique epitopes (upon multimerizing) that could be used as a basis to develop profilin multimer-based diagnostics and immunotherapeutics:

- 1) The form Vrtala injected into the animal models is not clear, but likely is a monomeric form. Conditions to make a soluble form were followed that would produce mostly monomeric profilin (in Methods: "The recombinant protein produced a single peak in the chromatogram obtained by high-pressure liquid chromatography and was completely soluble").
- 2) Production of a monomeric form (displayed in Figures 1 and 2) for injection is consistent with the production in animal models of monomeric-recognizing IgG and IgE (lesser degree) shown in Figure 3. Indeed, there were no noted

antibodies that recognized the larger profilin forms.

- 3) 20x of the profilin (Bet v2) was required vs. Bet v1 : Vrtala's assumption is because it's due to "some intrinsic property", but "there is currently no feasible experimental model to definitively prove this hypothesis" (page 920, last paragraph).

Considering information in the present application, the reason 20x more of Bet v2 profilin vs. Bet v1 was needed by Vrtala to elicit a response was because the injected solution contained monomers (i.e., weaker allergen/antigen) or undetectably small amounts of multimers such that a high concentration was needed to achieve an immune response.

Vrtala teaches away from the claimed invention. The form of rBet v2 injected into the animal models was likely monomeric. On page 914 in the Vrtala Methods section relied upon by applicant for this assertion, "the recombinant protein produced a single peak in the chromatogram" refers to the rBet v1 protein. There is not contrary data for rBet v2.

Therefore, Vrtala further illustrates contrasting conclusions to the present invention about the use of profilin multimers in diagnostics and therapeutics. The utility of profilin polymers was not recognized nor was it obvious that the profilin polymers would be a key allergen.

8. **Claims Appendix.**

See attached.

9. **Evidence Appendix.**

See attached.

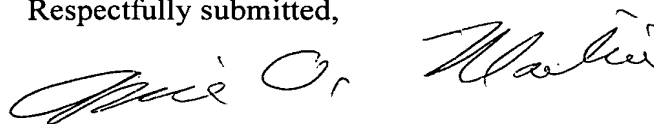
10. **Related Proceedings Appendix.**

None

\* \* \*

Applicant submits a check in the amount of \$250.00 to cover the filing fees for the appeal and a Petition for a Three-Month Extension of Time. It is respectfully requested that, if necessary to effect a timely response, this paper be considered as a Petition for an Extension of Time sufficient to effect a timely response and shortages in other fees, be charged, or any overpayment in fees be credited, to the account of Barnes & Thornburg LLP, Deposit Account No. 12-0913 (21511-92177).

Respectfully submitted,

A handwritten signature in cursive script, appearing to read "Alice O. Martin".

Alice O. Martin  
Registration No. 35,601

Dated: December 18, 2006  
Barnes & Thornburg LLP  
P.O. Box 2786  
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## 8. Claims Appendix

17. A diagnostic test for allergies, said test comprising:

- (d) obtaining a pharmaceutical composition of multimeric profilin;
- (e) administering the composition to a subject; and
- (f) determining a reaction from which allergenicity is inferred.

22. The method of claim 17, wherein multimeric profilin is selected from a group consisting of naturally occurring, synthetic, or recombinantly made profilin.

23. The diagnostic test of claim 17, wherein the profilin occurs as complexes of homomultimers.

24. The diagnostic test of claim 17, wherein the multimeric profilin comprises synthetic peptide fragments of profilin.

25. The diagnostic test of claim 24, wherein the multimeric profilin comprises synthetic peptide fragments that arise from profilin multimerization.

26. The diagnostic test of claim 17, wherein the multimeric profilin comprises peptide fragments made by recombinant DNA technology.

27. The diagnostic test of claim 17, wherein the multimeric profilin comprises monomers selected from the group consisting of celery (Api g4, GENE BANK ACCESSION NO. QPXF37) (SEQ ID NO: 1), peanut (Ara h5, GENE BANK ACCESSION NO. Q9SQ19) (SEQ ID NO: 2), birch tree pollen (Bet v2, GENE BANK ACCESSION NO. P25816) (SEQ ID NO: 3), Bermuda grass (Cyn d12, GENE BANK ACCESSION NO. 004725) (SEQ ID NO: 4), soybean (Gly m3, GENE BANK ACCESSION NO. 065809) (SEQ ID NO: 5), 065810 (SEQ ID NO: 6)), sunflower (Hel A2, GENE BANK ACCESSION NO. 081980) (SEQ ID NO: 7), latex (Hev b8, GENE BANK ACCESSION NO. CAB51914) (SEQ ID NO: 8), 065812 (SEQ ID NO: 9), Q9STB6 (SEQ ID NO: 10), Q9M7NO (SEQ ID NO: 11), Q9M7M9 (SEQ ID NO: 12), Q9M7M8 (SEQ ID NO: 13), Q9LE18 (SEQ ID NO: 14)), *Mercurialis annua* (Mer al, GENE BANK ACCESSION NO. 049894) (SEQ ID NO: 15), olive tree pollen (Ole e2, GENE BANK ACCESSION NO. P19963) (SEQ ID NO: 16), 0024170m (SEQ ID NO: 17) 024171 (SEQ ID NO: 18)), timothy grass (Phl pl 1, GENE BANK ACCESSION NO. P35079) (SEQ ID NO: 19), 024650 (SEQ ID NO: 20), 024282 (SEQ ID NO: 21)), sweet cherry (Pru av4, GENE BANK ACCESSION NO. Q9XF39) (SEQ ID NO: 22)), pear (Pyr c4, Q9XF27) (SEQ ID NO: 23)), corn pollen (Zea Pro I, GENE BANK ACCESSION NO. B35081) (SEQ ID NO: 24); Zea Pro II, GENE BANK ACCESSION NO. P35080 (SEQ ID NO: 25); ZMPro III, GENE BANK ACCESSION



NO. P35083 (SEQ ID NO: 26); ZmProIV, GENE BANK ACCESSION NO. 022655 (SEQ ID NO: 27); ZmProV, GENE BANK ACCESSION NO. Q9FR39 (SEQ ID NO: 28)), human (profilin I, GENE BANK ACCESSION NO. P07737 (SEQ ID NO: 29); Profilin II isoform 1, GENE BANK ACCESSION NO. NP 444252 (SEQ ID NO: 30); and Profilin II isoform GENE BANK ACCESSION NO.NP 002619 (SEQ ID NO: 31), or combinations thereof.

28. The diagnostic test of claim 17, wherein the composition comprises pharmaceutically acceptable carriers or diluents.

## 9. Evidence Appendix

Susanne Vrtala, *Induction of IgE Antibodies in Mice and Rhesus Monkeys with Recombinant Birch Pollen Allergins: Different Allergenicity of Bet v 1 and Bet v 2* (J Allergy Clin Immunol, Vol. 98, No. 5, Part 1 1996)

U.S. Patent No. 5,583,046 *Birch Pollen Allergen P14 for Diagnosis and Therapy of Allergic Diseases* (Issued December 10, 1996)

# Induction of IgE antibodies in mice and rhesus monkeys with recombinant birch pollen allergens: Different allergenicity of Bet v 1 and Bet v 2

Susanne Vrtala, PhD,<sup>a</sup> Peter Mayer, VMD,<sup>b</sup> Fatima Ferreira, PhD,<sup>c</sup> Markus Susani, PhD,<sup>d</sup> Alec H. Sehon, PhD,<sup>e</sup> Diëtrich Kraft, MD,<sup>a</sup> and Rudolf Valenta, MD<sup>a</sup> Vienna and Salzburg, Austria, and Winnipeg, Manitoba, Canada

**Background:** Serologic measurements with recombinant birch pollen allergens, rBet v 1 and rBet v 2 (birch profilin), have shown that more than 95% of patients allergic to tree pollen mount high levels of IgE against rBet v 1, whereas only approximately 10% of the patients display rather low levels of IgE against rBet v 2.

**Objective:** In this study an attempt was made to determine whether the different allergenicity of the major birch pollen allergen, rBet v 1, and a minor birch pollen allergen, rBet v 2, might be related to a different immunogenicity of the proteins as evaluated in experimental animal systems (mice and rhesus monkeys).

**Methods:** Purified recombinant allergens were injected into mice and rhesus monkeys with aluminum hydroxide as adjuvant for elicitation of specific IgE responses. Antibody responses to the allergens were detected by immunoblotting, and time courses of immune responses were measured by ELISA.

**Results:** In both animal models more than the 10-fold dose of rBet v 2 was required to induce IgE antibodies, and even then, the amount of specific IgE antibodies elicited with rBet v 1 was substantially higher than that induced by rBet v 2. It was noted that rBet v 2 formed stable polymers through disulfide bonds.

**Conclusion:** In two different animal models (mice and rhesus monkeys) the major birch pollen allergen, rBet v 1, induced substantially higher levels of IgE than rBet v 2. A reduced allergenicity of Bet v 2 caused by polymer formation would be in agreement with previous studies indicating reduced allergenicity of proteins on chemical polymerization. (*J Allergy Clin Immunol* 1996;98:913-21.)

**Key words:** Allergens, recombinant Bet v 1 and Bet v 2, allergenicity, immunization, mice, rhesus monkeys

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Despite increasing knowledge of the biologic functions of allergens, there is still little understanding of why certain proteins are more allergenic than others. Those allergens against which a high percentage ( $\geq 60\%$ ) of patients are sensitized and which mostly induce high titers of IgE have been designated as major allergens. In tree pollen allergy, Bet v 1 represents a major allergen of birch pollen inducing specific IgE antibodies in more than 95% of patients allergic to birch pollen,<sup>1</sup> whereas birch pollen profilin (Bet v 2) induces IgE antibodies in about only 10% to 20% of patients allergic to pollen,<sup>1-4</sup> showing, however, extensive cross-reactivities with profilins from pollen and food.<sup>2,3</sup>

**Abbreviations used**

PBS:	Phosphate-buffered saline
SDS-PAGE:	Sodium dodecylsulfate-polyacrylamide gel electrophoresis

In this study we investigated the possibility that the difference in allergenicity of Bet v 1 and Bet v 2 observed in patients with allergy<sup>1</sup> may be attributable to different immunogenicity and intrinsic molecular differences of these two allergens and might be reproduced in experimental animal models. The two allergens were recently expressed as recombinant proteins in *Escherichia coli*, and in vitro studies demonstrated that their immunologic and physicochemical (e.g., molecular weight, isoelectric point) properties were similar to those of their natural counterparts.<sup>3,5,6</sup>

It has been generally accepted that induction of specific IgE responses to diverse protein antigens in mice and rats may serve as a useful model for investigating the pathogenetic mechanism underlying IgE-mediated allergies, which in part can be transferred to human beings.<sup>7-11</sup> Moreover, immunization of mice with tiny doses of antigens adsorbed onto Al(OH)<sub>3</sub> was shown to elicit specific IgE responses in mice<sup>12-14</sup> with features typical of T<sub>H2</sub> immune responses<sup>15</sup> and comparable to those observed in patients with allergy.<sup>16</sup> In contrast, a T<sub>H1</sub> response can be elicited in mice by injection of antigens with Freund's complete adjuvant.<sup>17,18</sup>

Pure and soluble rBet v 1 and rBet v 2 were used for induction of specific IgE antibodies in mice and rhesus monkeys. For induction of anti-Bet v 2 IgE antibodies in mice and rhesus monkeys, the immunizing dose of profilin had to be 20-fold higher than that of Bet v 1; even then, the amounts of the Bet v 2-specific IgE antibodies were lower than those of the Bet v 1-specific IgE antibodies. It could be shown that rBet v 2 formed polymers through disulfide bonds, and it is hence suggested that the decreased allergenicity of rBet v 2 might be related to its tendency to polymerize. A similar capacity to form polymers was also noted for natural profilins from birch and other sources (unpublished data). Our findings fit the observation that chemical polymerization of allergens can switch T<sub>H2</sub> to T<sub>H1</sub> immune responses.<sup>19-22</sup>

**METHODS****Purification of recombinant forms of Bet v 1 and birch profilin (Bet v 2)**

Recombinant Bet v 1 was expressed in *E. coli* JM105<sup>1</sup> and purified to homogeneity according to the previously described protocol.<sup>6</sup> The recombinant protein produced a single peak in the chromatogram obtained by high-pressure liquid chromatography and was completely soluble. Recombinant birch profilin, rBet v 2, was expressed at high levels in plasmid pMW175 *E. coli* BL21 (DE3). The purification protocol described by Valenta et al.<sup>3</sup> was modified so that a more than 95% soluble protein was yielded.<sup>23</sup> Both proteins were tested for IgE- and antibody-binding capacity in ELISA and immunoblot assays and further tested for their capacity to release histamine from basophils of patients with allergy, as described previously.<sup>3,5,6</sup>

**Reducing and nonreducing sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), immunoblots, and blot overlays**

The tendency of recombinant Bet v 2 to form polymers through disulfide bonds under nonreducing conditions was demonstrated by SDS-PAGE, immunoblotting, and blot overlays. SDS-PAGE was done as described by Laemmli.<sup>24</sup> Five micrograms of rBet v 2 and rBet v 1, respectively, were dissolved in 10  $\mu$ l sample buffer with or without  $\beta$ -mercaptoethanol and subjected to SDS-PAGE. Gels were then stained with Coomassie Brilliant Blue (Bio-Rad, Richmond, Calif.) as previously described.<sup>25</sup> Higher molecular weight bands, which were assumed to represent Bet v 2 polymers, were probed with two different rabbit anti-profilin antibodies (RP1 and RP3).<sup>26</sup> The binding of rBet v 2 to itself was further investigated by blot overlays. Recombinant Bet v 2 was separated by SDS-PAGE and blotted onto nitrocellulose.<sup>27</sup> Nitrocellulose strips were then incubated with iodine 125-labeled rBet v 1 and rBet v 2 under reducing and nonreducing conditions, respectively. Each strip was incubated with radiolabeled protein (500,000 cpm) with or without the addition of  $\beta$ -mercaptoethanol (5 mmol/L) as described by Valenta et al.<sup>26</sup> Binding of <sup>125</sup>I-labeled proteins was visualized by autoradiography with Kodak x-OMAT films and intensifying screens (Kodak, Heidelberg, Germany).

**Immunization of mice and rhesus monkeys with rBet v 1 and rBet v 2**

Eight-week-old female BALB/c and B6D2F<sub>1</sub> mice were obtained from a local experimental animal care unit (Himberg, Austria). Animals were maintained in the animal care unit of the Institute of General and Experimental Pathology of the University of Vienna according to the local guidelines for animal care. Groups of mice were immunized by subcutaneous injection in the neck with 0.1  $\mu$ g or 1  $\mu$ g of rBet v 1 and 0.1  $\mu$ g, 1  $\mu$ g, or 20  $\mu$ g of rBet v 2 adsorbed to 100  $\mu$ l of AluGel-S

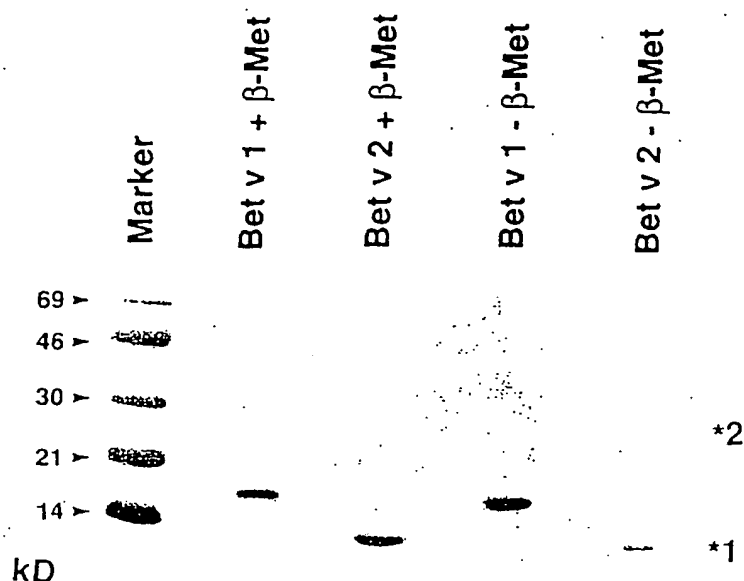


FIG. 1. Coomassie Brilliant Blue-stained SDS-PAGE showing purified recombinant Bet v 1 and recombinant birch profilin (Bet v 2) separated under reducing and nonreducing conditions. Five micrograms of purified recombinant Bet v 1 and 5  $\mu$ g of purified recombinant Bet v 2 were separated by SDS-PAGE under reducing (+ $\beta$ -mercaptoethanol) and nonreducing ( $-\beta$ -mercaptoethanol) conditions. Lane M is the molecular weight marker. Monomers (1) and dimers (2) in the nonreduced samples of Bet v 2 are indicated with asterisks.  $\beta$ -Met,  $\beta$ -Mercaptoethanol.

(Serva, Heidelberg, Germany) per mouse. A control group was immunized with 100  $\mu$ l of AluGel-S by using 0.9% sodium chloride instead of allergens. Immunizations were done with 30 mice of each of two strains (BALB/c and B6D2F1); groups of 10 mice were immunized with the same dose of antigen, and results were reproduced by using another 30 mice in a second experiment. Re-immunizations and bleedings were done at approximately 14-day intervals for a period of 9 months (in total 15 immunizations were done). Final bleedings were done after 1 year.

Groups consisting of two rhesus monkeys (one male and one female) were immunized subcutaneously with 100  $\mu$ l of AluGel-S in combination with allergen or phosphate-buffered saline (PBS) (negative control) monthly for a period of 6 months. Group I was immunized with 1  $\mu$ g of rBet v 1 per animal, group II received 10  $\mu$ g of rBet v 1 per animal, group III was injected with 1  $\mu$ g of rBet v 2 per animal, and group IV received 20  $\mu$ g of rBet v 2 per animal. The control group, consisting of one male monkey and one female monkey, was immunized with PBS without addition of allergen. In a second experiment four monkeys were immunized with 1  $\mu$ g of rBet v 1, and four monkeys received 20  $\mu$ g of rBet v 2. Monkeys were bled from the forearm 1 week before and 1 week after immunizations.

#### Determination of specific antibody responses by immunoblot and ELISA

Allergen-specific antibody responses were determined by immunoblotting and ELISA. For determination of

mouse antibody levels, ELISA plates (Nunc, Roskilde, Denmark) were coated with 100  $\mu$ l/well of the recombinant allergens diluted to 5  $\mu$ g/ml in PBS. The plates were washed two times with PBS-0.05% Tween and blocked for 2.5 hours with PBS-1% wt/vol bovine serum albumin-0.05% vol/vol Tween at room temperature; and 100  $\mu$ l/well of the mouse serum dilutions in PBS-0.5% bovine serum albumin-0.05% Tween was added (IgE, 1:20; IgG<sub>1</sub>, 1:2000; IgG<sub>2a</sub>, 1:200; IgG<sub>3</sub>, 1:200). The plates were incubated with sera in duplicate overnight at 4°C and washed five times with PBS-0.05% Tween, and bound mouse immunoglobulins were detected with 100  $\mu$ l of monoclonal rat anti-mouse immunoglobulin antibodies diluted 1:1000 in PBS-0.5% wt/vol bovine serum albumin-0.05% vol/vol Tween (PharMingen, San Diego, Calif.) overnight at 4°C. After it was washed five times with PBS-0.05% vol/vol Tween, a 1:2000 diluted horseradish peroxidase-coupled sheep anti-rat antiserum (Amersham, Buckinghamshire, U.K.) was added for 30 minutes at 37°C and 30 minutes at 4°C. Plates were again washed five times with PBS-0.05% Tween, and ABTS (60 mmol/L citric acid, 77 mmol/L Na<sub>2</sub>HPO<sub>4</sub> · 2H<sub>2</sub>O, 1.7 mmol/L ABTS [Sigma, St. Louis, Mo.], 3 mmol/L H<sub>2</sub>O<sub>2</sub>) was added. Plates were incubated in the dark for 30 minutes at room temperature, and the color reaction was stopped by addition of 100  $\mu$ l/well 0.32% NaF. Extinctions (optical density, 450 to 405 nm) were determined with an ELISA reader (Dynatech, Denkendorf, Germany). To exclude the possibility that blocking IgG antibodies can interfere with the IgE binding, sera

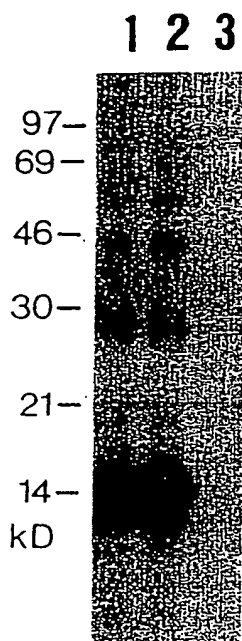


FIG. 2. Detection of Bet v 2 polymers with profilin-specific antisera. Recombinant Bet v 2 was separated by SDS-PAGE and blotted onto nitrocellulose under nonreducing conditions and detected with two different anti-profilin antisera (lane 1: RP1; lane 2: RP3). In lane 3 a normal rabbit serum was used as a negative control.

were also depleted for IgG<sub>1</sub> and IgG<sub>2a/b</sub> by preincubation with an anti-mouse IgG<sub>1</sub> and IgG<sub>2a/b</sub> monoclonal rat antibody (PharMingen) coupled to Sepharose 4B (Pharmacia, Uppsala, Sweden) (data not shown). IgE and IgG antibodies of immunized rhesus monkeys were determined by immunoblotting<sup>28</sup> and ELISA. Recombinant Bet v 1 and rBet v 2 (2 µg/cm gel) were separated by SDS-PAGE<sup>24</sup> and blotted onto nitrocellulose.<sup>27</sup> Nitrocellulose strips were incubated with 1:10 diluted monkey sera for IgE detection and 1:1000 diluted sera for IgG detection. Bound IgE and IgG were detected with rabbit anti-human IgE or IgG antisera (Dako, Glostrup, Denmark). Both antisera were shown to cross-react with rhesus monkey immunoglobulins and to be specific for IgE and IgG in a previous study.<sup>28</sup> To compare IgE levels of Bet v 1- and Bet v 2-immunized rhesus monkeys, ELISAs were done. The ELISA was performed as described for the mouse ELISA, except that IgE was detected with a rabbit anti-human IgE antiserum (Dako) and a horseradish peroxidase-labeled donkey anti-rabbit antiserum (Amersham).

## RESULTS

### Characterization of purified recombinant allergens, rBet v 1 and recombinant birch profilin (rBet v 2)

The investigation of the capacity of rBet v 1 and rBet v 2 to elicit a specific IgE response in vivo

required purification protocols delivering highly pure and soluble proteins, which contained no lipopolysaccharide or other contaminants. Recombinant Bet v 1 was purified from *E. coli* JM105 transformed with pKK 223-3 containing the Bet v 1 complementary DNA<sup>1</sup> according to the protocol described by Ferreira et al.<sup>6</sup> The cDNA coding for Bet v 2, which was originally expressed in *E. coli* JM105 pKK 223-3,<sup>1,2</sup> was transferred into plasmid pMW175 for high level expression in *E. coli* BL21 (DE3).<sup>23</sup> The recombinant protein was purified as previously described.<sup>3</sup> Pure and more than 95% soluble recombinant proteins were obtained, which according to different in vitro assays (binding of patients' IgE,<sup>1,3</sup> binding of specific antibodies,<sup>3,6</sup> capacity to release histamine from basophils of patients with allergy<sup>3,5,6</sup>) were found to behave similarly to the natural allergens. Lack of lipopolysaccharide and contaminants in the recombinant allergen preparations was determined by specific histamine release with human basophils and limulus assays (data not shown). Fig. 1 shows a Coomassie Brilliant Blue-stained SDS-polyacrylamide gel. Five micrograms of purified rBet v 1 and rBet v 2 were separated under reducing (+β-mercaptoethanol) and nonreducing conditions (−β-mercaptoethanol). Purified rBet v 1 produced one band at 17 kD under reducing and nonreducing conditions, whereas rBet v 2 migrated as a single band at 14 kD only under reducing conditions. When β-mercaptoethanol was omitted from the sample buffer, additional bands could be stained, of which one migrated at 28 kD. To further investigate whether the higher molecular bands that were observed in the Bet v 2 preparation under nonreducing conditions might be due to formation of polymers through disulfide bonds, immunoblots were done. Two different anti-profilin antisera identified the higher molecular weight bands as Bet v 2 polymers (Fig. 2). Blot overlays done with <sup>125</sup>I-labeled rBet v 2 showed specific binding of rBet v 2 to blot-immobilized rBet v 2, whereas no reaction with rBet v 1 (negative control) was observed. Under reducing (+β-mercaptoethanol) conditions, rBet v 2 did not bind to itself (data not shown). It is hence suggested that the binding occurs through the formation of disulfide bonds.

### Antibody responses induced with recombinant allergens in mice

Mice and monkeys that had been immunized with 1 µg rBet v 1 produced significantly higher

TABLE 1. Antibody responses of BALB/c mice immunized with recombinant Bet v 1 (1  $\mu$ g/injection) or Bet v 2 (20  $\mu$ g/injection)

	Time of first response (week $\pm$ SD)	Time of maximal response (week $\pm$ SD)	OD of maximal response ( $\pm$ SD)
Bet v 1			
IgE	10.6 ( $\pm$ 4.08)	18.4 ( $\pm$ 8.16)	0.6 ( $\pm$ 0.15)
IgG <sub>1</sub>	15.4 ( $\pm$ 3.21)	27 ( $\pm$ 4.36)	2.5 ( $\pm$ 0)
IgG <sub>2a</sub>	20.9 ( $\pm$ 5.46)	35.9 ( $\pm$ 11.63)	1.6 ( $\pm$ 0.84)
IgG <sub>3</sub>	16.9 ( $\pm$ 1.07)	27 ( $\pm$ 8.19)	0.27 ( $\pm$ 0.05)
Bet v 2			
IgE	20 ( $\pm$ 3.42)	30.3 ( $\pm$ 5.47)	0.19 ( $\pm$ 0.05)
IgG <sub>1</sub>	22.6 ( $\pm$ 6.43)	30.4 ( $\pm$ 4.79)	2.20 ( $\pm$ 0.57)
IgG <sub>2a</sub>	24 ( $\pm$ 2.65)	31.4 ( $\pm$ 4.39)	0.86 ( $\pm$ 0.91)
IgG <sub>3</sub>	15.7 ( $\pm$ 3.55)	28.3 ( $\pm$ 3.2)	0.19 ( $\pm$ 0.04)

Time-course and antibody responses of BALB/c mice immunized with recombinant Bet v 1 (1  $\mu$ g/injection) or Bet v 2 (20  $\mu$ g/injection). Mean values of the times when first significant antibody responses and maximal responses were measured in a group of seven Bet v 1-immunized mice and seven Bet v 2-immunized mice are displayed in weeks together with the standard deviation (SD). Mean values of antibody responses measured at the time of maximum immune response are displayed as optical density and SD. For the measurement of specific IgE, sera were diluted 1:20; for IgG<sub>1</sub>, 1:2000; for IgG<sub>2a</sub>, 1:200; and for IgG<sub>3</sub>, 1:200.

OD, Optical density.

levels of specific IgE, whereas in the case of rBet v 2, 20  $\mu$ g was necessary to induce specific IgE responses. No detectable IgE levels could be induced in mice and rhesus monkeys with 1  $\mu$ g of rBet v 2. The fact that the amounts of rBet v 2, which were necessary to induce detectable IgE responses in mice and rhesus monkeys, were 20-fold (20  $\mu$ g/animal) higher than the amounts of rBet v 1 (1  $\mu$ g/animal) needed, prompted us to investigate Bet v 1- and Bet v 2-specific antibody responses in detail. Table 1 shows the antibody responses in a group of seven mice immunized with 1  $\mu$ g of rBet v 1 and seven mice immunized with 20  $\mu$ g of rBet v 2 determined by ELISA. IgE, IgG<sub>1</sub>, IgG<sub>2a</sub>, and IgG<sub>3</sub> responses were measured over a period of 57 weeks at serum dilutions in which antigen was coupled in excess to antibodies to allow quantitation of results. Mice immunized with rBet v 1 showed an earlier rise of Bet v 1-specific IgE antibodies and mounted approximately threefold higher levels of specific IgE than the rBet v 2-immunized mice. The differences in the IgG responses were less pronounced. The rBet v 1-immunized mice displayed, for the most part, less than double the levels of specific IgG<sub>2a</sub> and IgG<sub>3</sub> antibodies compared with the rBet v 2-immunized mice, and comparable IgG<sub>1</sub> levels were reached.

To exclude the possibility that blocking IgG antibodies might influence IgE binding, additional control experiments were done. Sera were depleted for IgG<sub>1</sub> and IgG<sub>2a/b</sub> antibodies before IgE reactivity was measured, confirming that the Bet v

1-specific IgE titers were significantly higher than the Bet v 2 IgE responses. Comparable results were obtained in B6D2F<sub>1</sub> mice (data not shown).

#### Difference in antibody responses of rhesus monkeys to recombinant forms of Bet v 1 and birch profilin (Bet v 2)

The IgE and IgG reactivity of a representative Bet v 1-allergic monkey and a Bet v 2-allergic monkey with nitrocellulose-blotted purified rBet v 1 and rBet v 2 are displayed in Fig. 3. Sera obtained 1 week before and after each immunization were tested for IgE and IgG reactivity with nitrocellulose-blotted purified rBet v 1 (Fig. 3, A) and rBet v 2 (Fig. 3, B). As in the mouse system, a more intense binding to rBet v 1 was observed than to rBet v 2, whereas the IgG reactivity was comparable. Because the RAST-based immunoblot assay is a semiquantitative technique, the IgE levels induced with rBet v 1 and rBet v 2 in rhesus monkeys were investigated by ELISA experiments done at conditions of antigen excess to allow quantification of responses. Fig. 4 shows the ELISA determinations of specific IgE antibodies in five rBet v 1- and five rBet v 2-immunized monkeys after the fourth month of immunization (immunizations were done monthly). Only in one rBet v 2-immunized monkey (no. 7) could specific IgE be detected by ELISA, whereas in the sera of the rBet v 1-immunized monkeys (nos. 1 to 5) high levels of specific IgE could be measured. It must be stated, however, that the five rBet v 2-immunized monkeys had

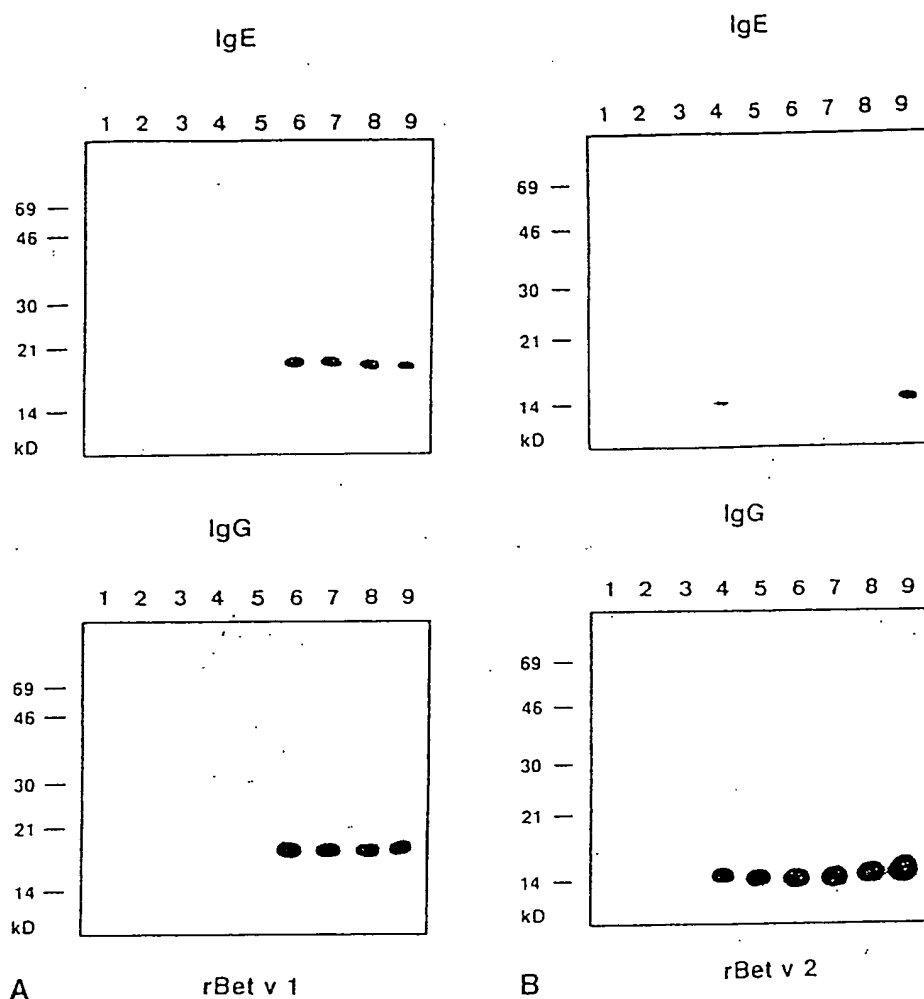


FIG. 3. IgE and IgG reactivity of a representative Bet v 1-immunized rhesus monkey (A) and a Bet v 2-immunized rhesus monkey (B) with nitrocellulose-blotted rBet v 1 and rBet v 2. For IgE detection, sera were diluted 1:10, and for IgG detection, 1:1000. To allow a comparison of signal intensity all blots were exposed for the same time (4 hours). In the first lane the monkeys' preimmune serum was used, whereas in the other lanes, strips were incubated with serum samples obtained 1 week before and after the monthly immunizations. The Bet v 1-immunized monkey had received 1  $\mu$ g of rBet v 1 per immunization, whereas the Bet v 2-immunized monkey was immunized with 20  $\mu$ g of rBet v 2 per injection.

positive rBet v 2-specific IgE when tested with the more sensitive immunoblotting technique and had mounted anti-rBet v 2 IgG titers comparable to those observed in the rBet v 1-immunized rhesus monkeys (data not shown).

#### DISCUSSION

Type I allergy represents a health problem of increasing importance in industrialized countries. Knowledge of the structures and sequences of allergens has increased tremendously because of the application of cDNA cloning techniques in the recent past.<sup>29</sup> Several allergens have been cloned

and sequenced and can be expressed by recombinant techniques.<sup>30,31</sup> Although biologic functions of several allergens are known or suspected, there is still a lack of knowledge about why certain proteins are more effective in eliciting an IgE response.

House dust mite allergens, such as Der p 1,<sup>32,33</sup> seem to have protease activity. The major birch pollen allergen Bet v 1<sup>34</sup> and the white-faced hornet venom allergen Dol m 5<sup>35</sup> are homologous to pathogenesis-related plant defense proteins, profilin (Bet v 2,<sup>2</sup> Phl p 11<sup>36</sup>) represents an actin-binding protein, and Bet v 3 belongs to a novel



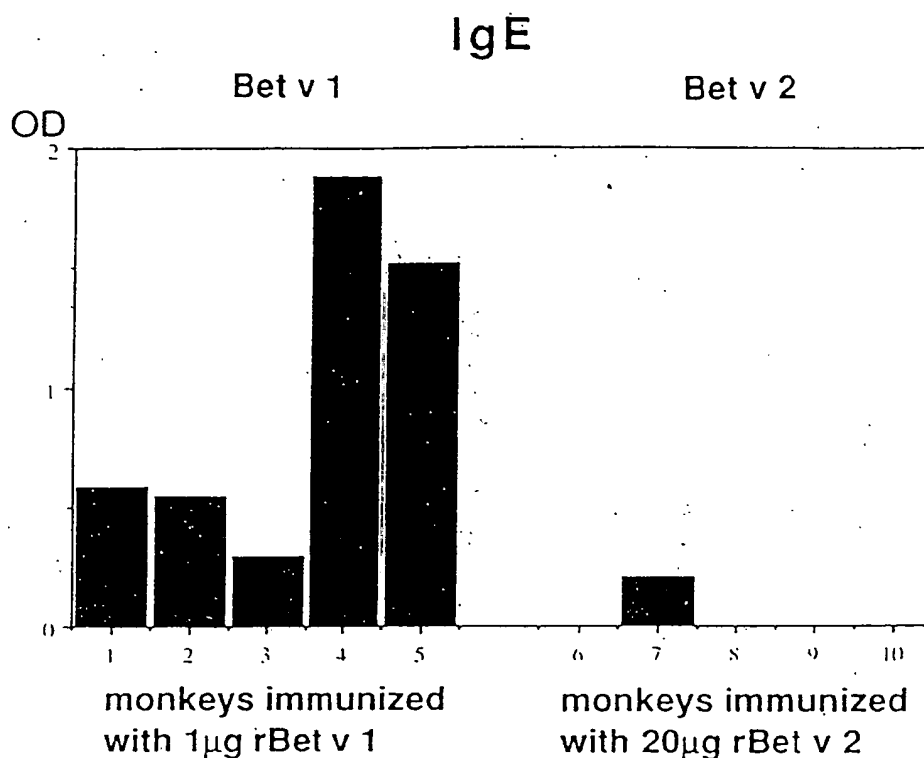


FIG. 4. ELISA measurements of Bet v 1- and Bet v 2-specific IgE antibodies in rhesus monkeys. Levels of Bet v 1- and Bet v 2-specific IgE were measured at serum dilutions of 1:5 in five rBet v 1-immunized rhesus monkeys (1 to 5) and in five rBet v 2-immunized monkeys (6 to 10). Extinctions are displayed as optical densities (OD) on the y axis. Numbers of the animals are displayed on the x axis.

class of calcium-binding proteins.<sup>37</sup> In the case of fungal allergens, homologies of Asp f 1 with mitogillin could be found.<sup>38</sup> The only obvious common properties of most allergens seem to be their low molecular weight (10 to 70 kd) and their rapid release from airborne particles on contact with aqueous solutions. Hence a relation between allergenicity and speed of release on hydration was discussed earlier.<sup>39</sup>

Another way to determine whether the differences in allergenicity of allergens might be linked to intrinsic properties of proteins is to study the immunogenicity of purified proteins in experimental animal systems. We have chosen two well-characterized pollen allergens, Bet v 1 and Bet v 2, which are now available as pure recombinant proteins possessing the same immunologic and physicochemical characteristics as the natural proteins. A greatly different percentage of patients is sensitized to Bet v 1 and Bet v 2. Bet v 2 was classified as a minor allergen because only 10% to 20% of patients show IgE reactivity, whereas more than 95% of patients allergic to birch pollen are sensi-

tized against Bet v 1, which therefore represents a major allergen.<sup>1</sup> Both allergens have a comparable molecular weight (17 kd and 14 kd, respectively) and are not significantly glycosylated. Recombinant Bet v 1 and Bet v 2 were expressed in *E. coli* as nonfusion proteins and were purified to homogeneity as soluble proteins, which possess immunologic and physicochemical properties similar to those of their natural counterparts. Purified rBet v 1 and rBet v 2 were injected at different doses into mice and rhesus monkeys by using Al(OH)<sub>3</sub> as adjuvant. Two different animal models were chosen to rule out differences in the immunogenicity of the proteins caused by genetic restrictions of the hosts.

In the mouse model, as well as in the rhesus monkey model, rBet v 1 induced significantly higher titers of specific IgE already at low doses (1 µg/animal) compared with rBet v 2, which induced IgE responses only at high amounts (20 µg/animal). The comparable difference in allergenicity observed in mice and rhesus monkeys strongly suggests that this difference is not limited to the

choice of a certain animal model or a restricted genetic background of the host. In fact, when we tested patients allergic to birch pollen, we found that a high proportion of these patients displayed IgG reactivity against Bet v 2 but produced no detectable levels of IgE antibodies.

As in the animal models, the levels of Bet v 1-specific IgE were substantially higher than those of Bet v 2-specific IgE.<sup>40</sup> Although the experimental animal models studied certainly differ in many aspects (e.g., use of recombinant allergens, use of adjuvant) from the process of natural sensitization in human beings, it is reasonable to assume that the different allergenicity of Bet v 1 and Bet v 2 might result in part from the different immunogenicity of these allergens.

The observation that Bet v 2 induced lower levels of IgE antibodies in human beings and in experimental animal models, although still substantial IgG responses were elicited, led us to suspect that the difference in allergenicity might also be linked to intrinsic properties of the allergens. When purified rBet v 1 and rBet v 2 were analyzed by SDS-PAGE under reducing and non-reducing conditions, we found that rBet v 2 formed polymers under nonreducing conditions. Chemical polymerization of antigens was already described to favor a T<sub>H1</sub> immune response in mice,<sup>19-22</sup> and it is hence possible that the weaker capacity of rBet v 2 to induce IgE antibodies might be linked to the ability of Bet v 2 to form natural polymers through disulfide bonds. Although it must be stressed that there is currently no feasible experimental model to definitely prove this hypothesis, the observation is in agreement with experimental data suggesting that polymerization of antigens might be a mechanism with which to reduce the allergenicity of protein antigens in favor of a T<sub>H1</sub> response.

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